VPI96-14CON(RCE) #11 BQ 10/17/02

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	David M. Naff
Group Art Unit	:	1651
Applicants	:	Alexey L. Margolin et al.
Application No.	:	09/631,241 Confirmation No.: 9677
Filed	:	August 2, 2000
For	:	CONTROLLED DISSOLUTION CROSSLINKED PROTEIN CRYSTALS

DECLARATION UNDER 37 C.F.R. § 1.132

I, BHAMI C. SHENOY, Ph.D., declare and state as follows:

1. I am a Principal Scientist at Altus Biologics Inc. ("Altus"), 625 Putnam Avenue, Cambridge, Massachusetts, 02139, the assignee of the above-identified United States patent application ("the '241 application"). I have held this position since 2001. From 1999 to 2000, I was a Staff Scientist at Altus and from 1997 to 1999, I held the position of Scientist at Altus. During this time, the focus of my research has been on protein and enzyme crystals, many in crosslinked form.

2. I was awarded a Ph.D. degree in Biochemistry from the University of Mysore, India in 1985. My Ph.D. thesis was entitled "Studies on Amylases with Special Reference to Glucoamylases - Purification and Characterization, Structure-function Studies, Immobilization, Chemical Modifications."

During the period between 1985 and 1997, I held the position of Senior Research Associate at Case Western Reserve University in Cleveland, Ohio. During the period between 1979-1985, I held research positions at Central Food Technological Research Institute, Mysore, India, including those of Research Fellow, Department of Biochemistry (1984-1985), Senior Research Fellow, Department of Biochemistry (1982-1984) and Junior Research Fellow, Department of Biochemistry (1979-1981). Further details of my educational and research career are set forth in my curriculum vitae, which is attached hereto as Exhibit 1.

3. I have spent over 20 years working with proteins, in particular enzymes, and studying their structure and function. In addition, I have extensive knowledge and experience in the area of biological synthesis, purification and characterization of proteins, particularly enzymes.

4. I have read the following documents: the '241 application (Exhibit 2); the April 1, 2002 Office Action issued in the '241 application (Exhibit 3); and Navia et al.'s United States patent 5,618,710 (the "Navia '710 patent", Exhibit 4). I make this declaration to demonstrate that crosslinked protein crystals prepared according to the Navia '710 patent do not display the dissolution characteristics of crosslinked protein crystals as described in the '241 application, i.e., crosslinked protein crystals crosslinked with a multifunctional crosslinking agent capable of controlled dissolution from insoluble and stable form to soluble and active form upon a change in the environment surrounding said crystals. More particularly, crosslinked protein crystals according to the Navia '710 patent remain intact when subjected to the same change in environment that causes controlled dissolution of the crosslinked protein crystals according to the '241 application.

5. As detailed below, I prepared crosslinked urease crystals ("Urease-CLEC™") according to the crosslinking conditions of Example 9 of the Navia '710 patent and crosslinked Candida rugosa lipase crystals ("CR-CLEC™") according to the crosslinking conditions of Example 10 of the Navia '710 patent. In addition, I used commercially available PeptiCLEC™-TR (Altus)

as a substitute for the Elastase-CLEC™ described in Example 4 of the Navia '710 patent.

6. As discussed below, I tested the dissolution behavior of Urease-CLEC™, CR-CLEC™ and PeptiCLEC™-TR at time points of 3 hours and 24 hours when incubated in 50 mM sodium phosphate (pH 7.5 or 9.0) at 25°C. These dissolution experiments are analogous to those reported for crosslinked Candida rugosa lipase crystals in Examples 24 and 25 of the '241 application. The result of these studies is the subject of this declaration.

7. I prepared crosslinked urease crystals according to Example 9 of the Navia '710 patent as follows: Fifteen thousand units (approximately 180 mg) of lyophilized Jack Bean urease (Boehringer Mannheim) were dissolved in 3 ml of 150 mM sodium phosphate buffer (pH 6.8). Acetone was added to the 3 ml urease solution by overnight vapor diffusion against 80 ml of 50% acetone/50% water. The solution was stirred gently during the addition of acetone. Crystallization was complete in sixteen hours and urease crystals were recovered by centrifugation (10 min at 1500 x *g*). The urease crystals were washed two times with 40% acetone in 50 mM sodium phosphate

buffer (pH 6.8). The crystals were recovered by centrifugation following each washing.

8. The urease crystals were crosslinked in a solution of 2% glutaraldehyde, 30% acetone in 50 mM sodium phosphate (pH 6) and washed with four, one liter volumes of 50 mM sodium phosphate buffer (pH 6.8). Washed crystals were suspended in deionized water and lyophilized.

9. I prepared crosslinked Candida rugosa lipase crystals according to Example 16 of the '241 application as follows: A 0.5 kg aliquot of Candida rugosa lipase ("CRL") in powder form (Meito) was mixed with 0.5 kg celite. The mixture was then placed in 10.2 L distilled deionized water to dissolve the CRL and subsequently, total volume adjusted to 20 L with the deionized water. The suspension was then mixed with an Air Drive Lightning Mixer for 2 hours at room temperature before being filtered through a 0.5 μm filter to remove celite. The mixture was then ultrafiltered and concentrated to 1.4 L (46.9 g) using a 3K hollow fiber filter membrane cartridge. Solid calcium acetate was added to a concentration of 5 mM calcium acetate. The pH was adjusted to pH 5.5 with concentrated acetic acid. A 0.7 L aliquot of the mixture was crystallized by either addition of 0.175 L of 2-methyl-2,4-pentanediol ("MPD") or of

0.35 L of a 30% solution of PEG-8000. The resulting solution was mixed and crystallization was allowed to proceed overnight at ambient temperature for about 17-20 hrs.

10. I prepared crosslinked Candida rugosa lipase crystals according to Example 10 of the Navia '710 patent as follows: A 45 µl aliquot of 50% untreated neat glutaraldehyde (Sigma) was added stepwise to 300 µl of stirred lipase crystals (6 mg/ml) in 20 mM Tris.HCl (pH 6.8), at room temperature. The final crosslinker concentration was 7.5%. Crosslinking was allowed to proceed over 30 min. and the crystals then recovered by low speed centrifugation and washed with 20 mM Tris.HCl (pH 6.8).

11. The term Candida rugosa lipase is a synonym in the art for the term Candida cylindracea lipase (see abstract of Stoddard Hatch et al., "Inhibition of yeast lipase (CRL1) cholesterol esterase (CRL3) by 6-chloro-2-pyrones: comparison with porcine cholesterol esterase," *Biochem. Biophys. Acta* 2002, 1596:381-391, Exhibit 5).

12. I also tested the dissolution properties of PeptiCLEC™-TR, a commercially-available crosslinked thermolysin crystal as a substitute for Elastase-CLEC™, as described in Example 4 of the Navia '710 patent. PeptiCLEC™-TR is an example

of a crosslinked enzyme crystal displaying the protease resistance of crosslinked enzyme crystals according to the Navia '710 patent. Thermolysin (MW 34,333 daltons) and elastase (MW 27,812 daltons) are proteases which belong to the same class of enzymes, metalloproteinases. Both contain zinc at the active site and have similar three-dimensional structure (see abstract of McKay et al., "Crystallographic structures of the elastase of Pseudomonas aeruginosa," *Matrix Suppl.* 1992, 1:112-115, Exhibit 6).

13. I carried out solubility tests of each of the Urease-CLEC™, CR-CLEC™ and PeptiCLEC™-TR crosslinked crystals at pH 7.5 and pH 9.0 as follows: The crystals were incubated at 1 mg/ml in 50 mM sodium phosphate (pH 7.5 or pH 9.0) containing 25% MPD. Aliquots were removed after 3 hr and 24 hr incubations at 25°C with stirring. Insoluble material was removed by filtration (0.25 μm). Soluble protein concentration was determined by measuring the absorbance at 280 nm. The results are presented in Table 1, below:

Table 1

CLEC™ Sample	[Protein] (mg/ml) in the supernatant at pH 7.5 after 3 hrs	[Protein] (mg/ml) (% of total protein) in the supernatant at pH 7.5 after 24 hrs	[Protein] (mg/ml) (% of total protein) in the supernatant at pH 9.0 after 3 hrs	[Protein] (mg/ml) (% of total protein) in the supernatant at pH 9.0 after 24 hrs
PeptiCLEC™-TR	0.0012	0.0015 (0.15%)	0.0021 (0.21%)	0.0025 (0.25%)
Urease-CLEC™	0.0000	0.0000 (0%)	0.0000 (0%)	0.0000 (0%)
CR-CLEC™	0.0000	0.0000 (0%)	0.0000 (0%)	0.0000 (0%)

14. Table 1 demonstrates that crosslinked protein crystals according to the Navia '710 patent are either totally insoluble or less than 0.5% soluble in 50 mM sodium phosphate containing 25% MPD over a period of 3 to 24 hrs at 25°C at pH 7.5 and pH 9.0. In my opinion, a dissolution level less than 1% indicates that the crosslinked protein crystals are essentially insoluble.

15. Tables 2 and 3 below illustrate the solubility results for crosslinked Candida rugosa lipase crystals according to Examples 24 and 25 of the '241 application, respectively, contrasted with the solubility results for the Urease-CLEC™, CR-CLEC™ and PeptiCLEC™-TR crosslinked crystals reported in Table 1. Crosslinked crystals were incubated at 1 mg/ml in 50 mM

sodium phosphate (pH 7.5 or pH 9.0) containing 25% MPD. Aliquots were removed after 3 hr and 24 hr incubations at 25°C with stirring. Insoluble material was removed by filtration (0.25 µm). Soluble protein concentration was determined by measuring the absorbance at 280 nm.

Table 2

Crosslinked Crystal	Final crosslinker concentration (%)	[Protein] (mg/ml) (% of total protein) at pH 9.0 after 3 hr	[Protein] (mg/ml) (% of total protein) at pH 9.0 after 24 hr
Example 18 of '241 Application	4 24 hrs lipase	0.47 (47%)	1 (100%)
Example 19 of '241 Application	4 lipase 24 hrs	0.60 (60%)	0.63 (63%)
Example 20 of '241 Application	4 lipase 24 hrs	0.42 (42%)	0.49 (49%)
PeptiCLEC™-TR		0.0021 (0.21%)	0.0025 (0.25%)
Urease-CLEC™	2	0.0000 (0%)	0.0000 (0%)
CR-CLEC™	7.5 lipase	0.0000 (0%)	0.0000 (0%)

Table 3

Crosslinked Crystal	Final crosslinker concentration (%)	[Protein] (mg/ml) (% of total protein) at pH 7.5 after 3 hr	[Protein] (mg/ml) (% of total protein) at pH 7.5 after 24 hr
Example 21 of '241 Application	4 lipase 3 hrs	0.12 (12%)	0.91 (91%)
Example 22 of	6.5 lipase 1 hr	0.63 (63%)	1.0 (100%)

'241 Application			
Example 23 of '241 Application	6.0	0.17 (17%)	0.69 (69%)
PeptiCLEC™-TR		0.0012 (0.12%)	0.0015 (0.15%)
Urease-CLEC™	2	0.0000 (0%)	0.0000 (0%)
CR-CLEC™	7.5	0.0000 (0%)	0.0000 (0%)

16. Table 2 demonstrates that crosslinked Candida rugosa lipase crystals prepared according to Examples 18-20 of the '241 application exhibit increasing dissolution over a 3 to 24 hr time period in 50 mM sodium phosphate at pH 9.0 containing 25% MPD. In particular, the crosslinked Candida rugosa lipase crystals of Example 18 were 47% soluble at 3 hr and 100% soluble at 24 hr. The crosslinked Candida rugosa lipase crystals of Example 19 were 60% soluble at 3 hr and 63% soluble at 24 hr and the crosslinked Candida rugosa lipase crystals of Example 20 were 42% soluble at 3 hr and 49% soluble at 24 hr. This is in contrast to the insolubility exhibited by the Urease-CLEC™ and CR-CLEC™ crosslinked crystals prepared according to the Navia '710 patent, assayed under the same dissolution conditions. The PeptiCLEC™-TR crosslinked crystals were also essentially insoluble (0.21% at 3 hr and 0.25% at 24 hr) under the same dissolution conditions.

17. Table 3 demonstrates that crosslinked lipase crystals prepared according to Examples 21-23 of the '241 application also exhibited increasing dissolution over a 3 to 24 hr time period in 50 mM sodium phosphate at pH 7.5 containing 25% MPD. For example, the crystals prepared according to the crosslinking conditions in Example 21 exhibited solubility of 12% at 3 hr and 91% at 24 hr. Crystals prepared according to Examples 22 and 23 displayed solubility of 63% and 17% at 3 hr and solubility of 100% and 69% at 24 hr, respectively. This is in contrast to the insolubility exhibited by the Urease-CLEC™ and CR-CLEC™ crosslinked crystals prepared according to the Navia '710 patent and measured under the same dissolution conditions. The PeptiCLEC™-TR crosslinked crystals were also essentially insoluble (0.12% at 3 hr and 0.15% at 24 hr) under the same dissolution conditions.

18. In my opinion, these experiments establish that crosslinked urease and lipase crystals prepared according to the Navia '710 patent, as well as the commercially available PeptiCLEC™-TR, are insoluble or essentially insoluble when placed in the same dissolution conditions as those set forth in Examples 24 and 25 of the '241 application. On the other hand, crosslinked protein crystals prepared according to Examples 18-

23 of the '241 application exhibit increasing dissolution over a 3 hr to 24 hr period under those conditions.

19. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that that such willful false statements may jeopardize the validity of the '241 application or any patent issued thereon.

B. C. Shenoy

/ BHAMI C. SHENOY, Ph.D.

Signed this 1st day of
October, 2002
at Cambridge, Massachusetts

CURRICULUM VITAE

Bhami C. Shenoy

HOME ADDRESS:

16 Westgate Drive, T-6
Woburn, MA 02161
781-937-6028

OFFICE ADDRESS:

Altus Biologics
625 Putnam Avenue
Cambridge, MA 02139
617-299-2941 (phone)
617-299-2999 (fax)

RESEARCH POSITIONS

1979-1981	Jr. Research Fellow, Biochemistry Department, Central Food Technological Research Institute, Mysore, India
1982-1984	Sr. Research Fellow, Biochemistry Department, Central Food Technological Research Institute, Mysore, India
1984-1985	Research Fellow, Biochemistry Department, Central Food Technological Research Institute, Mysore, India
1985- 1997	Sr.Research Associate, Department of Biochemistry/Biophysics and Physiology, Case Western Reserve University, Cleveland, Ohio
1997- 1999	Scientist, Altus Biologics, Inc. Cambridge, MA.
1999- 2000	Staff Scientist, Altus Biologics, Inc. Cambridge, MA.
2001-	Principal Scientist, Altus Biologics, Inc. Cambridge, MA.

EDUCATION:

Ph.D. in Biochemistry, 1985. *University of Mysore, India.*
Thesis: *Studies on Amylases with special reference to Glucoamylases—Purification and characterization, Structure-function studies, Immobilization, Chemical modifications* .

M.S. in Biochemistry, 1977. University of Mysore, India.
Worked on: *Polysaccharide-protein interactions in the neem gum (Azadirachta indica)*
B.S. in Botany, Zoology and Chemistry, 1974. *University of Mysore, India.*

PROFESSIONAL ORGANIZATIONS: American Chemical Society; American Association of Pharmaceutical Scientists

PUBLICATIONS:

1. Nayak, R., Shenoy, B.C. and Pattabiraman, T.N. (1979) Studies on plant gums: Role of calcium in polysaccharide-protein interaction in the neem (Azadirachta indica) gum. J.Biosci., **1**, 61-68.
2. Lakshman, M., Shenoy, B.C. and Raghavendra Rao, M.R. (1981) Purification and properties of diaminopimelate decarboxylase of *Micrococcus glutamicus*. J.Biosci., **3**, 89-104.
3. Manjunath, P., Shenoy, B.C. and Raghavendra Rao, M.R. (1983) Fungal glucoamylases. Part I. J.Applied Biochemistry, **5**, 235-260.
4. Shenoy, B.C., Appu Rao A.G. and Raghavendra Rao, M.R. (1984) Structure and stability of glucoamylase II from *Aspergillus niger* - A Circular Dichroism study. J.Biosci., **6**, 601-611.
5. Shenoy, B.C., Katwa, L.C., Appu Rao A.G. and Raghavendra Rao, M.R. (1985) Fungal glucoamylases. Part II. J.Biosci., **7**, 399-419.
6. B.C.Shenoy, A.G.Appu Rao and M.R.Raghavendra Rao. (1987) Effect of chemical modification on structure and activity of glucoamylases from Aspergillus candidus and Rhizopus species. J.Biosci., **11**, 339-350.
7. Ramadoss, C.S., Shenoy, B.C. and Borthakur, A. (1987) Isolation and characterization of a novel haemo-protein b-559 from bengal gram (*Cicer arietimum*). Biochem.J., **243**, 723-728.
8. Bhami C. Shenoy, Suvinay Paranjape, Vicki L. Murtif, Ganesh K. Kumar, David Samols and Harland G. Wood. (1988) Effect of mutations at Met-88 and Met-90 on the biotination of Lys-89 of the apo 1.3S subunit of Transcarboxylase. FASEB J., **2**, 2505-2511.
9. Bhami C. Shenoy and Harland G. Wood (1988) Purification and properties of the synthetase catalyzing the biotination of the aposubunit of transcarboxylase from Propionibacterium shermanii. FASEB J., **2**, 2396-2401.
10. T. Shanmugasundaram, Ganesh K. Kumar, Bhami C. Shenoy and Harland G. Wood. (1989) Chemical modification of the functional Arginine residues of carbon monoxide dehydrogenase from *Clostridium thermoaceticum*. Biochemistry, **28**, 7112 - 7116.
11. Shenoy, B.C., Xie, Y., Park, V.L., Kumar, G.K. Beegen, H., Wood, H.G. and Samols, D. (1992) The importance of methionine residues for the catalysis of the biotin enzyme, transcarboxylase: Analysis by site directed mutagenesis. J. Biol. Chem., **267**, 18407-18412.
12. Hsieh, P-C., Shenoy, B.C., Jentoft, J.E. and Phillips, N.F.B. (1993) Purification of Polyphosphate and ATP glucokinase phosphotransferase from Mycobacterium tuberculosis $H_{37}Ra$: Evidence that Poly(P) and ATP glucokinase activities are catalyzed by the same enzyme. Protein Exp. and Purification, **4**, 76-84.

13. Shenoy, B.C., Kumar, G.K. and Samols, D. (1993) Dissection of the biotinyl subunit of transcarboxylase into regions essential for activity and assembly. J. Biol. Chem., **268**, 2232-2238.
14. Shenoy, B. C., Magner, W., Kumar, G.K., Phillips, N.F.B., Haase, C.F. and Samols, D. (1993) The Non-biotinylated form of the 1.3 S subunit of transcarboxylase binds to Avidin (monomeric)-agarose: Purification and separation from the biotinylated 1.3 S subunit. Protein Exp. and Purification, **4**, 85-94.
15. Hsieh, P-C.; Shenoy, B.C.; Haase, F.C.; Jentoft, J.E. and Phillips, N.F.B. (1993) Involvement of Tryptophan(s) at the active site of Polyphosphate/ATP glucokinase from Mycobacterium tuberculosis. Biochemistry, **32**, 6243-6249.
16. Shenoy, B.C.; Samols, D. and Kumar, G.K. (1993) The conserved methionines of the 1.3 S biotinyl subunit of transcarboxylase: Effect of mutations on conformation and activity. Arch. Biochem. Biophys., **304**, 359-366.
17. Xie, Y., Shenoy, B.C., Magner, W.J., Hejilik, D.P. and Samols, D. (1993) Purification and Characterization of the Wild type 5S subunit of Transcarboxylase Expressed in *E. coli.*, Protein Exp. and Pur., **4**, 456-464.
18. Woo, S.B.., Shenoy, B.C., Wood, H.G., Magner, W.J., Kumar, G.K., Beegen, H. and Samols, D. (1993) Effect of deletion from the carboxyl terminus of the 12 S subunit on activity of Transcarboxylase. J.Biol.Chem., **268**, 16413-16419.
19. Shenoy, B.C., Xie, Y., Sha, D. and Samols D. (1993) Identification and Characterization of a Factor which is essential for assembly of Transcarboxylase. Biochemistry, **32**, 10750-10756.
20. Thorton, C.G., Kumar, G.K., Carl Haase, F., Phillips, N.F.B., Woo, S.B., Park, V.L. Magner, W.J., Shenoy, B.C., Wood, H.G. and Samols, D. (1993) Primary structure of the monomer of the 12S subunit of transcarboxylase as deduced from DNA and characterization of the product expressed in *Escherichia coli.* J. Bacter. **175**, 5301-5308.
21. Thorton, C.G., Kumar, G.K., Shenoy, B.C., Carl Haase, F., Phillips, N.F.B., Park, V.M., Magner, W.J., Hejilik, D.P., Wood, H.G. and Samols, D. (1993) Primary structure of the 5 S subunit of transcarboxylase as deduced from the genomic DNA sequence. FEBS Lett. **330**, 191-196.
22. Eswaran, D., Ali, M.S., Shenoy, B.C., Korotchkina, L.G., Roche, T.E., and Patel, M.S. (1995) Arginine-239 in the beta subunit is at or near the active site of bovine pyruvate dehydrogenase Biochem.Biophys. Acta, **1252**, 203-208.
23. Ali, M.S., Shenoy, B.C. Eswaran, D., Andersson, L.A., Roche, T.E., and Patel, M.S. (1995) Identification of the tryptophan residue in the thiamine pyrophosphate binding site of mammalian pyruvate dehydrogenase. J.Biol.Chem., **269,** 4570-4574.
24. Hsieh, P-C., Shenoy, B.C., Samols, D. and Phillips, N.F.B. (1996) Molecular Cloning, expression and Characterization of Polyphosphate Glucokinase from *Mycobacterium tuberculosis*. J. Biol. Chem., **271,** 4909-4915.
25. O'Connor, M.J., Dunlap, R.B., Odom, J.D., Hilvert, D., Carey, M.P., Shenoy, B.C. and Carey, P.R. (1996) Probing an Acyl Enzyme of Selenosubtilisin by Raman Spectroscopy. J. Amer.Chem. Soc., **118,** 239-240.

26. Reddy, D.V., Shenoy, B.C., Carey, P.R. and Sonnichsen, F.D. (1997) Absence of observable biotin-protein interactions in the 1.3S subunit of transcarboxylase: An NMR study. Biochemistry **36**, 8501-8512.
27. Reddy, D.V., Rothemund, S., Shenoy, B.C., Carey, P.R. and Sonnichsen, F.D. (1998) Structural chracterization of the entire 1.3S subunit of transcarboxylase from Propionibacterium shermanii. Protein Science, **7,** 2156-2163.
28. St Clair N, Shenoy B, Jacob LD, Margolin AL. (1999) Cross-linked protein crystals for vaccine delivery. *Proc Natl Acad Sci U S A*, **96**, 9469-74
29. Dinakarpandian D, Shenoy BC, Hilvert D, McRee DE, McTigue M, Carey PR (1999), Electric fields in active sites: substrate switching from null to strong fields in thiol- and selenol-subtilisins. *Biochemistry,* **38**, 6659-67
30. Reddy DV, Shenoy BC, Carey PR, Sonnichsen FD (2000) High resolution solution structure of the 1.3S subunit of transcarboxylase from Propionibacterium shermanii. Biochemistry, **39**, 2509-16.
31. Shenoy B, Wang Y, Shan W, Margolin AL (2001) Stability of crystalline proteins. *Biotechnol Bioeng.*,**73**, 358-69